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Nitrous Oxide Emissions from Biofilm Processes for Wastewater Treatment

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Abstract

This paper discusses the microbial basis and the latest research on nitrous oxide (N_2O) emissions from biofilms processes for wastewater treatment. Conditions that generally promote N_2O formation in biofilms include (1) low DO values, or spatial DO transitions from high to low within the biofilm; (2) DO fluctuations within biofilm due to varying bulk DO concentrations or varying substrate concentrations; (3) conditions with high reaction rates, which lead to greater formation of intermediates, e.g., hydroxylamine (NH_2OH) and nitrite (NO_2^-), that promote N_2O formation; and (4) electron donor limitation for denitrification. Formation of N_2O directly results from the activities of ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and heterotrophic denitrifying bacteria. More research is needed on the roles of AOA, comammox, and specialized denitrifying microorganisms. In nitrifying biofilms, higher bulk ammonia (NH_3) concentrations, higher nitrite (NO_2^-) concentrations, lower dissolved oxygen (DO), and greater biofilm thicknesses result in higher N_2O emissions. In denitrifying biofilms, N_2O accumulates at low levels as an intermediate, and at higher levels at the oxic/anoxic transition regions of the biofilms and where COD becomes limiting. N_2O formed in the outer regions can be consumed in the inner regions if COD penetrates sufficiently. In membrane-aerated biofilms, where nitrification takes place in the inner, aerobic biofilm region, the exterior anoxic biofilm can serve as a N_2O sink. Reactors that include variable aeration or air scouring, such as denitrifying filters, trickling filters, or rotating biological contactors (RBCs), can form peaks of N_2O emissions during or following a scouring or aeration event. N_2O emissions from biofilm processes depend on the microbial composition, biofilm thickness, substrate concentrations and variability, and reactor type and operation. Given the complexity and difficulty in quantifying many of these factors, it may be difficult to accurately predict emissions for full-scale treatment plants. However, a better understanding of the mechanisms, and the impacts of process configurations, can help minimize N_2O emission from biofilm processes for wastewater treatment.

Keywords: N_2O, biofilms, hydroxylamine, MBBR, MABR, MBfR, granules

INTRODUCTION

Wastewater treatment processes can be a significant source of nitrous oxide (N_2O), a powerful greenhouse gas (GHG) with a global warming potential around 300 times that of carbon dioxide (CO_2) (Montzka et al. 2011). N_2O is very stable, and may persist in the atmosphere for over 120 years (Kampschreur et al. 2009; Schreiber et al. 2012). The U.S. Environmental Protection Agency (EPA) estimates that U.S. wastewater treatment plants emit around 5.2 Tg N_2O yr^{-1} as CO_2 equivalents (Ritter 2014), and these amounts are expected to increase with time (Law et al. 2012; Okabe et al. 2011).

Much past research has addressed N_2O emissions from suspended growth processes (Ahn et al. 2010; Kampschreur et al. 2009; Law et al. 2012). However, much less is known about emissions from biofilm processes, such as the moving bed biofilm reactor (MBBR), integrated fixed-film activated sludge (IFAS), biological aerated filter (BAF), granular sludge, and membrane-aerated biofilm reactors (MABRs) (Henze et al. 2008; Martin and Nerenberg 2012; Syron and Casey 2008). Biofilm processes are becoming increasingly popular due to their higher volumetric treatment rates, reduced operational costs, minimal need for settling, and operational simplicity (Henze et al. 2008; Khan et al. 2013; Nicolella et al. 2000; WEF 2010).

While the microbial basis of N_2O formation, i.e., the microorganisms and metabolic pathways leading to its formation, are the same for suspended-growth and biofilm systems, the observed behavior may be very different. This results from the microbial stratification, microbial interactions, substrate gradients, and substrate interactions unique to biofilms, as well as the biofilm reactor configuration (Henze et al. 2008; Law et al. 2012; Vlaeminck et al. 2010a). Thus, the "mechanisms" leading to N_2O emissions in biofilms may significantly differ from those of suspended growth systems.

Todt and Dorsch (2016) provided a comprehensive review of N_2O emissions from biofilm systems. They explored the biochemistry of N_2O production/consumption in relevant organisms, discussed current biofilm models, evaluated possible environmental factors affecting N_2O emissions, and tabulated emission factors for different processes. Massara et. al (2017) briefly addressed biofilms as part of a comprehensive review of N_2O emissions from wastewater processes. This review provides an update, considering new information on the N_2O emissions

from microbial systems. It also discusses new types of microbial metabolism and different biofilm reactor configurations, and their impacts on N_2O emissions.

BIOFILMS VS. SUSPENDED-GROWTH SYSTEMS

Biofilms are aggregates of microbial cells embedded in a network of self-produced extracellular polymeric substances (EPS) (Flemming et al. 2016; Stoodley et al. 2002). Biofilms are widespread in natural systems (Donlan 2002), and increasingly used in engineered treatment processes, especially for those with low substrate concentrations and high flows (Henze et al. 2008; Nicolella et al. 2000; WEF 2010). Unlike with suspended bacteria, diffusion and reaction in biofilms lead to substrate gradients. As a result, concentrations in the biofilm may differ significantly from those in the bulk liquid (Fig. 1). In addition, bacteria stratify into layers, where different types of metabolism may predominate at different depths within the biofilm.

FIGURE 1

The dynamics of growth, decay, and detachment influence the microbial community structure of biofilms (Elenter et al. 2007). Slow growing organisms may be "pushed out" of the biofilm by faster growing organisms (Lackner et al. 2008; Xavier et al. 2005). Metabolic products may diffuse out of the biofilm or may be consumed by other populations. pH gradients may form due to proton-producing or consuming processes within the biofilm (Vroom et al. 1999). The greater complexity of biofilms, compared to suspended growth processes, makes their behavior more difficult to predict.

N_2O AND NITROGEN CYCLE

This section discusses basic microbial transformations that affect N_2O formation in wastewater treatment processes. These processes are relevant to both suspended growth and biofilm processes. The relationship between these transformations and N_2O formation in biofilms is discussed in subsequent sections.

The nitrogen cycle includes a number of N species and both microbial and abiotic transformations, where N varies in redox state between -3 and +5. While most of the nitrogen cycle is well established, new biotic and abiotic transformation processes continue to be discovered (Daims et al. 2016; Kuypers et al. 2018; Schreiber et al. 2012; Stein and Klotz 2016). Figure 2 schematically shows key N species and biological transformations. For wastewater treatment processes, the key transformations include nitrification and denitrification, where nitrate (NO_3^-) is sequentially reduced to nitrogen gas (N_2). Both processes can lead to N_2O formation.

FIGURE 2

N_2O from Microorganisms Related to Nitrification

Nitrification is carried out by the sequential activity of ammonia-oxidizing bacteria (AOB) and archaea (AOA), and nitrite-oxidizing bacteria (NOB). AOB and AOA oxidize ammonia (NH_3) to nitrite (NO_2^-), with hydroxylamine (NH_2OH) as an intermediate (Fig. 3) (Daims et al. 2016; Guo et al. 2017), while NOB oxidize NO_2^- to NO_3^-. AOB directly produce N_2O through two main pathways: nitrifier denitrification and NH_2OH oxidation (Fig. 3). NOB, AOA, anammox, and comammox microorganisms may play an indirect role in N_2O formation by affecting the availability of NH_3 and NO_2^-.

FIGURE 3

In the nitrifier denitrification pathway, AOB reduce NO_2^- to nitric oxide (NO) and N_2O (Chandran et al. 2011; Kampschreur et al. 2007; Kim et al. 2010; Tallec et al. 2006) (Fig. 3). The NH_2OH oxidation pathway involves the oxidation of NH_2OH to NO by hydroxylamine oxidoreductase (HAO) and subsequent reduction to N_2O catalyzed by the enzyme NO reductase (Chandran et al. 2011; Law et al. 2012; Stein 2011) (Fig. 3).

Recent findings show that, in the canonical nitrifying bacteria *N. europaea*, two other routes for N_2O production exist under anaerobic conditions. One is the direct oxidation of NH_2OH to N_2O by cytochrome P460 (Caranto et al. 2016) and the nitrification intermediate NO (Caranto and Lancaster 2017). Although not all AOB share the same route for N_2O production, these recent

findings expand on previous knowledge where chemical reactions were thought to be mainly important at higher oxygen (O_2) levels (Liu et al. 2017a).

N_2O can also be produced biologically or abiotically by coupling NH_2OH oxidation with the reduction of NO_2^- (Harper et al. 2015; Terada et al. 2017), free nitrous acid (HNO_2) (Soler-Jofra et al. 2016), or NO (Spott et al. 2011). These are termed N-nitrosation hybrid reactions, or simply "hybrid" reactions (Spott and Stange 2011). In addition, metals such as copper (Harper et al. 2015) and manganese (Heil et al. 2015) can catalyze abiotic N_2O production from NH_2OH via the hybrid reaction. Under some conditions, the hybrid reaction can become a predominant pathway for N_2O production in a partial nitrifying reactor (Soler-Jofra et al. 2018; Terada et al. 2017). N_2O production via the hybrid reaction is enhanced in the presence of AOB (Liu et al. 2017a; Terada et al. 2017).

Under aerobic conditions, N_2O is mainly formed via the NH_2OH pathway, and rates are relatively low. When DO concentrations decrease, the nitrifier denitrification pathway becomes more important, leading to higher rates of N_2O formation (Chung and Chung 2000; Kampschreur et al. 2009; Ma et al. 2017a; Park et al. 2000; Tallec et al. 2008). However, under complete anoxic conditions N_2O emissions are again low due to the lack of DO for NH_3 oxidation (Fig. 3). Spikes of N_2O production can occur at transitions from anoxic to aerobic, or aerobic to anoxic, conditions, due to an electron imbalance (Domingo-Felez et al. 2014; Kampschreur et al. 2008; Sabba et al. 2015; Yu et al. 2010). Thus, N_2O emissions can be significant in processes with anoxic/aerobic stages or intermittent aeration (Chandran et al. 2011).

Unlike AOB, which have well elucidated N_2O production pathways, the pathways for AOA are yet to be fully understood (Blum et al. 2018b). They perform NH_3 oxidation in a similar way to AOB (Kozlowski et al. 2016); however, they lack the ability to produce N_2O enzymatically through side reactions of NH_3 oxidation or nitrifier denitrification, as mediated by AOB (Spang et al. 2012; Tourna et al. 2011; Walker et al. 2010). Stieglmeier et al. (2014) showed that *Nitrososphaera viennensis*, a pure culture of AOA from soil, produces N_2O via a hybrid reaction. While AOA are found in WWTPs (Park et al. 2006; Sauder et al. 2012; Zhang et al. 2009), AOA are more common in marine environments (Santoro et al. 2011) and soils (Gubry-Rangin et al. 2010; Li et al. 2018; Nicol et al. 2008; Zhang et al. 2012).

Anammox bacteria convert NH_3 and NO_2^- to N_2 under anoxic conditions (Kuypers et al. 2003). NO is a key intermediate in anammox metabolism (Kartal et al. 2011), and genomic

evidence suggests that anammox species have the potential to produce N_2O via NO reduction (Kartal et al. 2007; Strous et al. 2006). However, research suggests that N_2O production under process-relevant conditions is negligible (Blum et al. 2018a). Anammox may indirectly affect N_2O formation by heterotrophs and AOB by reducing the concentrations of NH_3 and NO_2^-.

Comammox bacteria are a subset of the genus *Nitrospira* capable of complete ammonia oxidation (comammox) via oxidation of NH_3 to NO_3^- (Daims et al. 2015; van Kessel et al. 2015). Comammox are thought to have a competitive advantage over conventional ammonia oxidizers (e.g. AOA and AOB) under ammonia-limiting conditions (Costa et al. 2006; Daims et al. 2015; Kits et al. 2017; van Kessel et al. 2015). While little is known about comammox in wastewater biofilms, van Kessel et al. (2015) and Daims et al. (2015) obtained comammox enrichments in the lab by operating their systems with low NH_3 concentrations. Thus, it is likely they play a role in wastewater biofilms under similar conditions.

Evidence suggests that comammox *Nitrospira*, as opposed to canonical *Nitrospira*, harbor genomic NH_3 and NO_2^- oxidation machinery homologous to classical AOB and NOB, respectively (e.g., gene clusters encoding *amo*, *hao*, and *nxr*) (Daims et al. 2015; van Kessel et al. 2015). However, very little is known about their capacity for N_2O production. NH_2OH appears to be an obligate intermediate of comammox metabolism, analogous to AOB catabolism, and it is likely that N_2O can be formed by comammox via the NH_2OH pathway (Fig. 3). Comammox genomes recovered to date also harbor capacity for NO_2^- reduction to NO (NirK), similar to non-comammox *Nitrospira* (Camejo et al. 2017; Lawson and Lucker 2018). Comammox clades A and B genomes reported to date lack a known NOR or proteins related to NOx metabolism (Palomo et al. 2018), similarly to common *Nitrospira* taxa (Lawson and Lucker 2018) and therefore may be incapable of nitrifier denitrification. Thus, the presence of reactive nitrogen species produced by comammox biomass, e.g. NO or NH_2OH, could to lead to abiotic reactions with the production of N_2O as a final product.

Comammox may be detrimental to PN/A systems, where NO_2^- production is needed. However, they may also reduce N_2O emissions by minimizing NO_2^- accumulation. The presence of comammox in wastewater treatment processes, both in suspended growth and biofilm processes, and the metabolic versatility of *Nitrospira* species including the two comammox *Nitrospira* clades is currently an active area of research. Future research should also address the selecting factors

for partitioning between comammox and canonical *Nitrospira*, and clarify the potential role for comammox in N_2O emissions.

N_2O from Microorganisms Related to Denitrification

Denitrification is the sequential reduction of NO_3^- and NO_2^- to NO, N_2O, and finally N_2 (Ni and Yuan 2015). It involves four enzymes: the nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (NOS). A schematic of the denitrification metabolism is shown in Figure 3.

The formation of N_2O in wastewater denitrification processes is often due to selective inhibition of the NOS enzyme (Guo et al. 2017). This can be caused by its greater sensitivity to DO (Firestone et al. 1979; Tallec et al. 2008), pH (Firestone et al. 1979; Hanaki et al. 1992), NO_2^- (Alinsafi et al. 2008), carbon source type and concentration (Tallec et al. 2006), carbon limitation (Alinsafi et al. 2008; Tallec et al. 2006), and hydrogen sulfide (H_2S) (Schonharting et al. 1998).

While denitrifying bacteria produce N_2O during denitrification, they also can reduce N_2O to N_2 (Read-Daily et al. 2016). Externally supplied N_2O can be reduced concurrently with NO_3^- and NO_2^- (Conthe et al. 2018; Pan et al. 2015; Pan et al. 2013a; Read-Daily et al. 2016).

While many denitrifying bacteria have a complete reduction pathway and can reduce NO_3^- and NO_2^- all the way to N_2, less is known about bacteria that can grow with N_2O but not with NO_3^- or NO_2^-. Newly classified clade II-type *nosZ* N_2O reducing bacteria were recently discovered (Jones et al. 2013; Sanford et al. 2012). These have since been detected in a granular sludge reactor (Lawson et al. 2017), a membrane-aerated biofilm reactor (MABR) (Kinh et al. 2017b) and a biofiltration system (Yoon et al. 2017). Some isolates harboring clade II type *nosZ* have higher affinity for N_2O reduction than those harboring clade I type *nosZ* (Suenaga et al. 2018; Yoon et al. 2016) whereas a contradictory finding was reported (Conthe et al. 2018), requiring more in-depth analysis concerning bacteria as an N_2O sink at a low N_2O concentration. Some clade II type *nosZ* bacteria appear to lack genes encoding for NIR and/or NOR, suggesting their potential as an N_2O sink but not an N_2O source (Graf et al. 2014). As reviewed elsewhere, these non-denitrifying N_2O-reducing bacteria in wastewater engineering are yet to be explored in detail (Hallin et al. 2018). The ecophysiology of non-denitrifying N_2O reducers in a biofilm system warrants further research.

There are a wide range of denitrifying microorganisms, and some with special behavior

with respect to N_2O formation and reduction. Some can fully reduce NO_3^- and NO_2^- to NH_3 in an ecologically important process called dissimilatory nitrate or nitrite reduction to ammonium (DNRA) (Stein and Klotz 2016) (Fig. 2). In this process, NO_3^- or NO_2^- is reduced to NH_3, with N_2O produced at the NO_2^- reduction stage as a by-product (Fig. 2) (Kelso et al. 1997; Rutting et al. 2011; Streminska et al. 2012). Unlike denitrification, this process conserves N in the ecosystem (Rutting et al. 2011; Tiedje et al. 1982). Many DNRA microorganisms can produce N_2O as a by-product (Stevens and Laughlin 1998; Stevens et al. 1998). Some of these microorganisms employ DNRA as a detoxification mechanism in order to avoid high concentration of NO_2^- (Kaspar 1982). However, the actual contribution of DNRA to N_2O formation in these species remains uncertain (Butterbach-Bahl et al. 2013).

Behavior regarding N_2O emissions may also vary based on the type of electron donor. For example, elemental-sulfur (S^o) oxidizing denitrifiers (Di Capua et al. 2015; Liu et al. 2017b), methane (CH_4) oxidizing denitrifiers (He et al. 2018), phosphate-accumulating (PAO) denitrifiers (Gao et al. 2017; Wang et al. 2011; Wang et al. 2014; Zhou et al. 2012), H_2 oxidizing denitrifiers (Li et al. 2017), and bacteria growing with an electrode as an electron donor (Jiang et al. 2018) display different behavior with respect to N_2O emissions. Methane-oxidizing denitrifiers appear to reduce NO_2^- to N_2 without forming N_2O as an intermediate, and therefore are thought to minimize N_2O emissions (He et al. 2018). While the details on each of these donors are beyond the scope of this review, the kinetics for each donor can have important impacts on N_2O formation and consumption.

TYPES OF BIOFILM REACTORS AND IMPACTS ON N_2O EMISSIONS

This section describes different type of biofilm reactors, and their special characteristics as relate to N_2O emissions. Based on the analysis in the previous section, and also following Todt et al. (2016) and Massara et al. (2017), conditions that promote N_2O emission include (1) low DO values, or DO spatially transitioning from high to low within the biofilm, as this leads to nitrifier denitrification or incomplete heterotrophic denitrification; (2) conditions where the DO fluctuates temporally from high to low values, (3) conditions with high reaction rates, which lead to greater formation of intermediates (e.g., NH_2OH, NO_2^-) that promote N_2O formation; and (4) limiting electron donor for denitrification.

The above factors may have different impacts for different types of biofilm reactors. There is a wide range of biofilm reactors, and they can be classified based on the arrangement of their solid, liquid, and gas phases, whether the carriers are fixed or moving, their carrier specific surface area (area of carrier per unit volume of reactor), their mixing regime (completely mixed or plug flow), and the mechanisms of transfer of gases and electron donor or acceptor substrates. Typical biofilm reactor configurations are shown schematically in Figure 4.

FIGURE 4

Trickling filters (Fig. 4A) are commonly used for COD removal and nitrification. The media is non-submerged, and is kept aerobic by convective air currents within the bed. While considered aerobic, anoxic niches can form in the deeper biofilm (Dalsgaard and Revsbech 1992). The variations in DO and donor concentration in the biofilm between passes of the wastewater distributor arm can lead to N_2O emissions. When used for nitrification, N_2O is likely to form within the bed, with some stripped by the air currents and present in the effluent (Melse and Mosquera 2014). There is little experimental data on N_2O emissions from trickling filters, possibly due to the difficulty in capturing the off-gases, and further research is needed in this area.

Biofilters (Fig. 4A) are similar to trickling filters, but used to treat gaseous contaminants such as odorous compounds in air or volatile organic compounds (VOCs). Air is passed through a non-submerged packed bed with biofilms growing on the media, and the contaminants partition into the liquid phase coating the biofilm. Yoon et al. (2017) proposed using a biofilter supplied to remove N_2O in off gases from an activated sludge aeration basin. Raw wastewater was used as the electron donor. In lab tests, 99.9% of N_2O was removed when supplied at 100 ppmV in N_2, i.e., without any O_2. However, removals decreased significantly when supplied in air. Biofilters are likely an expensive approach to mitigating N_2O emissions, as they require covering aeration basin to collect off gases, treating large volumes of gas, and adding an additional process and complexity to the treatment train.

Packed bed reactors (Fig. 4B and 4C) are fully submerged fixed bed biofilm reactors. They can be operated in upflow or downflow mode, and either aerated (e.g., for nitrification) or unaerated with electron donor addition (denitrifying filters). Upflow packed bed reactors, such as nitrifying or denitrifying filters, typically operate in plug flow fashion. Thus, the filters experience

high substrate concentrations at the influent end and low concentrations at the effluent end. The concentration gradients (e.g., high NH_3 at influent, low DO at effluent) can impact N_2O formation processes. When used for denitrification, air pulses are periodically performed at the bottom of the filter to release N_2 bubbles accumulating in the reactor. These pulses can strip N_2O formed at the beginning of the bed, when normally it would be reduced to N_2 further within the bed (Bollon et al. 2016). Whenever air is added to a denitrifying filter, there is potential for N_2O formation at some location within the biofilm due to the greater sensitivity of N_2OR to O_2 inhibition. N_2O may also accumulate due to insufficient electron donor supply. For nitrifying and denitrifying packed bed reactors, backwashing is carried out regularly to remove excess biomass. Thinner biofilms may not allow full treatment, leading N_2O breakthrough from the reactor. For denitrifying biofilms, breakthrough can also be caused by donor limitation. Bollon et al. (2016) found that a full-scale denitrifying filter with a C/N of 3 or higher had up to 93% N_2O reduction. However, during a carbon supply failure removals lowered 26%. Similar results were found by Capodici et al. (2018) and Zhang et al. (2016). In the latter study, the authors found that a decrease of the C/N from 3 to 0.65 led to an increase of the genes encoding for NOR that would enhance the transformation of NO to N_2O and lead to increased N_2O emissions. Zhang et al. (2017) studied the behavior of lab-scale denitrification filters and found a complex interaction of the denitrification with anammox and DNRA. Gene abundance, together with accumulation of NO_2^- at temperatures between 5 and 15 °C, were found important factors for N_2O accumulation. Further research is required to investigate the impact of influent NO_2^- and possible adaptation of bacteria to variable influent loadings of both NO_2^- and NO_3^- in denitrifying filters.

RBCs (Fig. 4D) use rotating wheels of media partially submerged in wastewater. When the wheels are outside the water, the biofilm can experience O_2 concentrations in the biofilm exterior, while the DO concentrations can drop significantly when immersed in the wastewater (Pynaert et al. 2002). This cycling of high and low DO concentrations, as well variations in donor concentration when the biofilm is submerged vs. when it is out of the wastewater, can potentially lead to higher N_2O emissions. There does not appear to be any published findings of N_2O emissions from RBCs. Note that RBCs are often covered to prevent from UV toxicity and to protect from low temperatures in winter. In these cases, it may be possible to pump air from the enclosures through an anoxic zone or into a biofilter, such as that described above, to reduce N_2O to N_2.

Airlift, MBBRs, and IFAS (Fig. 4E and 4G) use carriers that "float" in the water, and therefore have little relative velocity between the carrier and the water. They can be operated under aerobic or anoxic conditions. In continuous systems, the biofilm carriers are kept in a single zone, experiencing consistent bulk environments. This can avoid the high N_2O emissions in suspended growth systems transitioning from anoxic to aerobic zones (Chandran et al. 2011). Recent research on N_2O emissions from MBBRs are consistent with the factors described at the beginning of this section, depending on the application (Mannina et al. 2018a; Mannina et al. 2017; Mannina et al. 2018b; Wei et al. 2017).

Fluidized bed reactors (Fig. 4F) behave similarly to a BAF, but use much finer media. This provides a high specific surface area, and allows the particles to become suspended in the upward wastewater flow. These reactors also experience a somewhat higher degree of mixing, compared to packed bed reactors, but still have some plug flow behavior. Excess biofilm is continuously removed by abrasion, and biofilms typically are thinner than in BAFs. The behavior with respect to N_2O emissions should be similar to the BAFs. Note that aerobic granular sludge can behave similarly to a fluidized bed reactor. However, granular sludge is typically operated in sequencing batch mode (Castro-Barros et al. 2015). Recent research on N_2O emission from granular sludge also confirm the above mechanisms (Jia et al. 2018; Lu et al. 2018; Peng et al. 2017; Reino et al. 2017).

Counter-diffusional biofilms are those where one substrate diffuses from the bulk liquid, while the other penetrates the biofilm from the attachment surface. The counter-diffusion of substrates leads to a range of different behaviors with respect to conventional, co-diffusional biofilms (Nerenberg, 2016). Examples of counter-diffusional biofilms include MABRs, where the membranes are used to supply air or O_2; membrane-biofilm reactors (MBfRs) where membranes supply H_2 or CH_4 (Liu at al., 2017b); sulfur-based biofilms, where solid S^o particles support a biofilm (Wang et al. 2016a); and even bioelectrochemical biofilms (Jiang et al., 2018). MABR behavior is discuss in more detail in the next section.

MECHANISMS OF N_2O FORMATION IN BIOFILM PROCESSES FOR WASTEWASTER TREATMENT

Because of their special layered structure and organization, biofilms allow unique niche formation with specific metabolic functions. In addition, intermediates formed in one biofilm location can diffuse to another with different environments, leading to transformations that would not normally occur in a suspended growth system (Dalsgaard et al. 1995; de Beer 1997; Nielsen et al. 1990; Sabba et al. 2017b; Schreiber et al. 2009). This section discusses basic behavior of biofilms for some key processes, including nitrification, denitrification, combined nitrification and denitrification, and partial nitrification/anammox. The behavior is common for most biofilm reactors except for MABRs, which are described separately. The figures in this section are intended to illustrate typical behavior. They are only schematics, not meant to reflect an actual operating condition.

Nitrifying biofilms

Nitrifying biofilms form when NH_3 is the dominant or sole electron donor. While AOB and NOB are primary population members in nitrifying biofilms, heterotrophic bacteria typically co-exist (Kindaichi et al. 2004), growing on the decay products from nitrifying microorganisms (Gieseke et al. 2005; Okabe et al. 2005). However, N_2O production in nitrifying biofilms is likely dominated by AOB, with a minor contribution from heterotrophic bacteria. In this section, we focus on the mechanisms of N_2O from the nitrifying population. In the subsequent section, we discuss the impact of heterotrophs on nitrifying biofilms, especially when organic carbon is present in the bulk.

Typical substrate profiles in nitrifying biofilms, and zones of N_2O formation and emission, are shown schematically in Figure 5. In conventional, co-diffusional biofilms, the outer biofilm is aerobic and has the highest NH_3 concentrations. As a result, the NH_3 oxidation rates are high, leading to high NH_2OH concentrations. In addition, the nitrifier denitrification pathway is inhibited by the high DO in this zone. Thus, the NH_2OH oxidation pathway is likely to dominate, and N_2O formation rates are likely to be relatively low. Nitrifier denitrification may become significant in the aerobic/anoxic transition zone (Mao et al. 2008; Schreiber et al. 2009; Schreiber

et al. 2008). In the anoxic zone, N_2O formation rates are low. This is because NH_3 oxidation, which is the source of electrons for nitrifier denitrification, requires O_2. However, Sabba et al. (2015) proposed that NH_2OH formed in the aerobic biofilm exterior would diffuse to the interior anoxic zones. AOB in this zone could utilize NH_2OH as a rich electron source, enabling the nitrifier denitrification pathway and resulting in a spike of N_2O. Further research is needed to confirm this mechanism experimentally. In Figure 5, the N_2O concentration profile slopes towards the outer biofilm, indicating diffusive mass transfer towards the bulk. If diffused aeration is used, the N_2O is readily stripped from the liquid phase (Law et al. 2012; Rassamee et al. 2011; Wu et al. 2014).

Membrane-aerated biofilms (MABs) are a novel biofilm process for wastewater treatment, where O_2 is supplied from the membrane and NH_3 from the bulk (Martin and Nerenberg 2012; Syron and Casey 2008) (Fig. 5b). Because of the unique penetration of NH_3 and O_2 from opposite sides of the biofilm, they are called, as mentioned above counter-diffusional biofilms (Nerenberg 2016). N_2O can also occur in MABRs systems. In MABs, the highest nitrification rates usually occur in the biofilm interior, not at the outer edge. Thus, N_2O formation via the NH_2OH pathway is likely to occur in the deep biofilm. In addition, the aerobic/anoxic transition occurs in the biofilm interior, and the bulk is anoxic. Thus, while N_2O can be stripped from suspended growth systems by bulk aeration (Law et al. 2012; Rassamee et al. 2011; Wu et al. 2014), N_2O in MABRs can be consumed by denitrifying bacteria in the outer biofilm or bulk liquid. Conversely, some N_2O may be stripped from MABR biofilms by air flowing through the membrane lumen, if operated with open end membranes (Kinh et al. 2017a). Stripping from the lumen is indicated in Figure 5b by the slope of the N_2O concentration profile towards the membrane in its proximity.

FIGURE 5

NOB can contribute indirectly to N_2O emissions by scavenging DO and favoring the formation of a steeper gradient for transitioning from oxic to anoxic conditions (Sabba et al. 2017a; Sabba et al. 2015). They also can play a key role in reducing the NO_2^- concentration, which reduces the rates of nitrifier denitrification (Schreiber et al., 2009). Anammox bacteria can play a similar role in decreasing N_2O emissions (Pellicer-Nacher et al. 2010). As mentioned previously, NOB

do not play a direct role for NO and N_2O emissions, but may affect emission by modifying the NO_2^- concentrations (Wang et al. 2016b).

Denitrifying biofilms

Denitrifying biofilms are those where NO_3^- is the primary electron acceptor. We also consider biofilms with an aerobic exterior and denitrifying interior, but neglect any nitrification in the aerobic zone. In denitrifying biofilms, N_2O is an obligate intermediate. It is typically present at higher concentrations in the outer biofilm region, where NO_3^- and NO_2^- reduction activity is higher, but can diffuse and be consumed in deeper regions where NO_3^- and NO_2^- concentrations are lower (Fig. 6a). Thus, biofilms can have regions that can serve as an N_2O sink, mitigating N_2O emissions (Dalsgaard and Revsbech 1992; Nielsen et al. 1990).

FIGURE 6

In the presence of high DO, denitrification is usually inhibited and therefore little N_2O is formed (Fig. 6b). However, biofilms typically have DO gradients, and denitrification and N_2O formation may occur deeper in the biofilm (Dalsgaard and Revsbech 1992; Nielsen et al. 1990). In the transition zone from oxic to anoxic, higher amounts of N_2O will be formed due to the higher sensitivity of NOS to O_2 inhibition (Bonin et al. 1992; Lu and Chandran 2010; Morley et al. 2008; Otte et al. 1996). When this transition zone is near the outer biofilms, more N_2O may be exported to the bulk liquid. When the transition occurs deeper in the biofilm, i.e., at higher bulk DO concentrations, and when electron donor is sufficient, N_2O is more likely to be reduced in the deeper biofilm and less emissions will occur (Dalsgaard and Revsbech 1992).

If N_2O is formed in the outer biofilm, and if sufficient electron donor is available in the deeper zones of the biofilm, denitrifying biofilms can serve as an N_2O sink (Eldyasti et al. 2014; Sabba et al. 2017b). However, if sulfate reduction occurs in the deeper biofilm where NO_3^- has been depleted, H_2S may accumulate and inhibit N_2O reduction (Pan et al. 2013b). Electron donor limitation in the denitrifying zone also may result in greater N_2O formation (Dalsgaard and Revsbech 1992; Nielsen et al. 1990; Todt and Dorsch 2015) (Fig. 6c).

Combined nitrifying/denitrifying biofilms

Biofilms exposed to both organic carbon and NH_3 usually have an outer layer dominated by fast-growing heterotrophic bacteria (Henze et al. 2008). In the presence of non-limiting organic substrates, O_2 is usually consumed by heterotrophic activity with little formation nitrifying biomass. However, in presence of low or transient organic carbon concentrations, nitrifying organisms can develop in the biofilm. These biofilms are here referred as "combined nitrifying/denitrifying biofilms".

In combined nitrifying/denitrifying biofilms, the mechanisms of N_2O formation can be quite complex. Both co- and counter- diffusional combined nitrifying/denitrifying biofilms are characterized by the presence of complex communities where N_2O is formed by both nitrifiers and denitrifiers, but also reduced by denitrifiers (Matsumoto et al. 2007; Nerenberg 2016). Various intermediates play roles in both pathways, as indicated in Figure 2. For example, NO_2^- and NO, two crucial components of both nitrifier denitrification and NH_2OH oxidation pathways, also play a role as intermediates in the denitrification pathway (Todt and Dorsch 2015). Thickness is also a crucial component for both co- and counter- diffusional biofilm, if adequate thickness and COD concentrations are present, then N_2O reduction can occur (Eldyasti et al. 2014; He et al. 2017).

Co-diffusional combined nitrifying/denitrifying biofilms receive both electron donor and acceptor from the bulk (Fig. 7a). In this type of biofilm, heterotroph are typically more abundant in the outer biofilm, due to their faster growth rates and the greater availability of COD. This zone is typically aerobic, so little or no denitrification or N_2O reduction occurs. Nitrifiers are typically located in the aerobic zone below the heterotrophs. If enough COD is present, then N_2O reduction can occur in the deeper biofilm (Fig. 7a) (Chae et al. 2012; Eldyasti et al. 2014; He et al. 2017). When the bulk is aerated in co-diffusional combined nitrifying/denitrifying biofilms, there is greater N_2O mass transfer towards the bulk rather than towards the anoxic zone where it can be reduced. This translates in higher N_2O emissions.

FIGURE 7

In counter-diffusional combined nitrifying/denitrifying biofilms, DO penetrates the biofilm from the attachment surface. In this case, and assuming the bulk liquid is anoxic, the nitrifiers

would only be active near the membrane surface (Kinh et al. 2017a). In addition, N_2O formed by the nitrifiers could potentially be reduced by the heterotrophs in outer, anoxic region of the biofilm, where the COD concentrations are highest (Cole et al. 2004; Kinh et al. 2017b; LaPara et al. 2006). As seen for nitrifying biofilms (Fig. 5b), there could also be N_2O stripping by the membrane, as indicated from a negative slope of the N_2O profile towards the membrane (Fig. 7b). The lack of bulk aeration reduces N_2O mass transfer to the bulk. Note that MABR membranes can also strip CO_2 from the biofilm, leading to pH shifts that can impact the microbial community and potentially impact N_2O emissions (Ma et al. 2017b).

Based on the above, the type of biofilm (co- vs. counter- diffusional) also can affect the microbial community structure and therefore the N_2O emissions. For each bulk substrate condition and detachment regime, there may be a different microbial community structure, which in turn can affect the formation/reduction and emissions of N_2O. Therefore, the behavior of these biofilms is complex and hard to predict (Martin and Nerenberg 2012; Nerenberg 2016).

Partial nitritation/anammox biofilms

In combined partial nitritation/anammox (PN/A) reactors, NH_3 is partially oxidized to NO_2^- by AOB. The remainder of the NH_3 is then oxidized to N_2 gas via NO_2^- reduction by anammox bacteria. NOB are undesirable in PN/A reactors, and diverse strategies are employed to outselect these organisms. PN/A reactors typically also harbor a diverse flanking community, many of which are capable of heterotrophic denitrification (Lawson et al. 2017).

A distinguishing feature of PN/A systems is the presence of multiple biological sinks for NO_2^-. Biofilm-based PN/A systems are further distinguished by strong spatial segregation of AOB (in oxic layers) and anammox and denitrifiers (in anoxic, usually deep, layers) (Hubaux et al. 2015; Laureni et al. 2016; Okabe et al. 2011). Crossfeeding within the biofilm and capacity of certain denitrifiers to act as internal N_2O sinks, likely differentiates N_2O emissions in biofilms from suspended growth PN/A processes.

The potential of PN/A systems to act as significant N_2O sources, particularly from biofilm or hybrid PN/A reactors, is poorly understood. Results suggest that emissions depend strongly on bulk O_2 concentration (Harris et al. 2015), NO_2^- concentration (Van Hulle et al. 2012), NH_3 oxidation activity (Blum et al. 2018a; Domingo-Felez et al. 2014), nitrogen loading (Yang et al.

2016), aeration regime (intermittent vs. continuous aeration) (Blum et al. 2018a; Domingo-Felez et al. 2014; Kampschreur et al. 2008; Ma 2018), presence of organic matter (Jia et al. 2018), and biofilm thickness (Vlaeminck et al. 2010b).

Intermittent aeration mirrors conditions recently shown to promote N_2O generation (Chandran et al. 2011; Kampschreur et al. 2008; Kampschreur et al. 2009; Yu et al. 2010), but has also been suggested that appropriate intermittent aeration can facilitate control or minimization of N_2O emissions from PN/A processes (Castro-Barros et al. 2015; Domingo-Felez et al. 2014; Su et al. 2017).

While sources of N_2O in PN/A systems are still not well understood, multiple studies have indicated it may derive predominantly from AOB. Ali et al. (2016) provided evidence based that nitrifier denitrification and NH_2OH pathways were equally important to N_2O formation in the oxic surface region of granules from a PN/A reactor. However, ~30% of N_2O emissions in this system could be attributed to the anammox dominated anoxic interior of granules due to either heterotrophic denitrification or a yet unidentified pathway. Harris et al. (2015) showed that N_2O site preference data from a suspended growth PN/A reactor was inconsistent with current understanding of N_2O production pathways, and further suggested that N_2O emissions in this system could be due in part to an unknown inorganic or anammox-associated N_2O production pathway. In general, biofilm-based PN/A processes appear to emit less N_2O than suspended nitrifying processes (Gilmore et al. 2013). Further research is needed to better identify sources of N_2O in biofilm-based and hybrid biofilm suspended growth PN/A systems, and to quantitatively evaluate how spatial structuring, biofilm thickness, and aggregate architecture influence N_2O emissions in these emerging low energy N removal systems.

CONCLUSIONS

N_2O formation is promoted when there are (1) low DO values, or DO spatially transitioning from high to low within the biofilm; (2) conditions where the DO fluctuates temporally from high to low values; (3) conditions with high reaction rates, which lead to greater formation of intermediates (e.g., NH_2OH and NO_2^-) that promote N_2O formation; and (4) limiting electron donor for denitrification. The microbial basis of N_2O formation in biofilms and suspended growth systems are similar, yet N_2O emissions in biofilm systems depend greatly on microbial

stratification, the formation of substrate gradients, the exchange of intermediates within the biofilm, and the type of biofilm reactor. This can lead to different patterns and quantities of N_2O emission for the same bulk environment, and make it more difficult to predict N_2O emissions. Co-diffusional and membrane-aerated biofilms may have substantially different behavior, due to the unique microbial and stratifications and substrate profiles. In order to predict N_2O emissions from biofilm processes, and develop strategies to minimize them, it is important to understand the microbiological and biochemical basis for N_2O formation, the factors affecting N_2O formation in biofilms, as well as the impacts of reactor configurations and operating modes. Future research should address the pathways and kinetics of N_2O emissions from AOA, comammox bacteria, methane-oxidizing denitrifying bacteria, and others. It also is important to explore their abundance in biofilms. Given the complexity of biofilms and biofilm processes, empirical assessments of N_2O emissions from the broad range of biofilm reactors type and operating conditions is needed, and application-specific recommendations to minimize emissions should be developed.

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Compliance with Ethical Standards

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Conflict of Interest:

F. Sabba declares he has no conflict of interest.

A. Terada declares he has no conflict of interest.

G. Wells declares he has no conflict of interest.

B. F. Smets declares he has no conflict of interest.

R. Nerenberg declares he has no conflict of interest.

Ethical approval:

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure Captions

Fig. 1 Idealized schematics of (a) a floc, and (b) a biofilm. The biofilm schematic shows the liquid diffusion layer (LDL), as well as profiles of a substrate and metabolic product. Note that real flocs are highly complex and heterogeneous in morphology, and biofilms may have rough or dendritic surfaces with internal pores.

Fig. 2 Key processes in the N-cycle. N_2O is highlighted in gray (adapted from Daims et al. 2016 and Schreiber et al. 2012). The dashed line for comammox shows the formation of NO_2^- as intermediate but also its oxidation to NO_3^- by the same organism. Abbreviations in figure: DNRA is dissimilatory nitrite reduction to ammonia; assimil. is assimilatory; dissimil. is dissimilatory. Note that denitrification can produce N_2O, but it is also the only known process that can reduce it.

Fig. 3 Nitrogen transformations in AOB, NOB and DNB. Abbreviations: AOB, ammonia-oxidizing bacteria; NOB, nitrite-oxidizing bacteria; DNB, denitrifying bacteria, AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase (hydroxylamine dehydrogenase in *Nitrospira*); NXR, nitrite oxidoreductase; NirK, copper-containing nitrite reductase; NirS, cytochrome cd1 type nitrite reductase; NOR, nitric oxide reductase; and NOS, nitrous oxide reductase. Purple arrows show intermediates potentially shared between nitrification and denitrification pathways. Abiotic reactions (gray) are further discussed in the text.

Fig. 4 Types of biofilm reactors. (A) Unsubmerged filter (e.g., trickling filter or biofilter), (B) upflow fixed-bed reactor (e.g., biologically active filter (BAF), (C) downflow fixed-bed reactor (e.g., BAF), (D) rotating biological contactor (RBC), (E) suspended or airlift biofilm reactor, (F) fluidized-bed biofilm reactor (FBBR or granular sludge), (G) moving-bed biofilm reactor (MBBR), integrated fixed film activated sludge (IFAS), and (H) membrane-supported biofilm reactor (e.g., MBfR or MABR). Note: *i* = influent; *e* = effluent; *r* = recycle; *w* = wasting flow; *g* = gas flow (typically air) in or out. Black dots in figures E, F, and G are biofilm carriers. Adapted from (Morgenroth 2008) and (WEF 2010)

Fig. 5 N_2O formation in nitrifying biofilms. (a) Co-diffusional and (b) counter-diffusional. Solid black arrow indicates N_2O loss towards either bulk or membrane lumen. NO_2^- and NO are not shown for clarity.

Fig. 6 N_2O formation in denitrifying biofilms. (a) Excess e^- donor, (b) excess e^- donor with O_2, and (c) limiting e^- donor. Solid black arrow indicates N_2O loss towards bulk and dashed black arrow indicates reduction within the biofilm depth. NO_2^- and NO are not shown for clarity.

Fig. 7 N_2O formation in combined nitrifying/denitrifying biofilms. (a) Co-diffusional and (b) counter-diffusional. Solid black arrow indicates N_2O loss towards either bulk or membrane lumen; dashed black arrow indicates reduction within the biofilm depth. NO_2^- and NO are not shown for clarity

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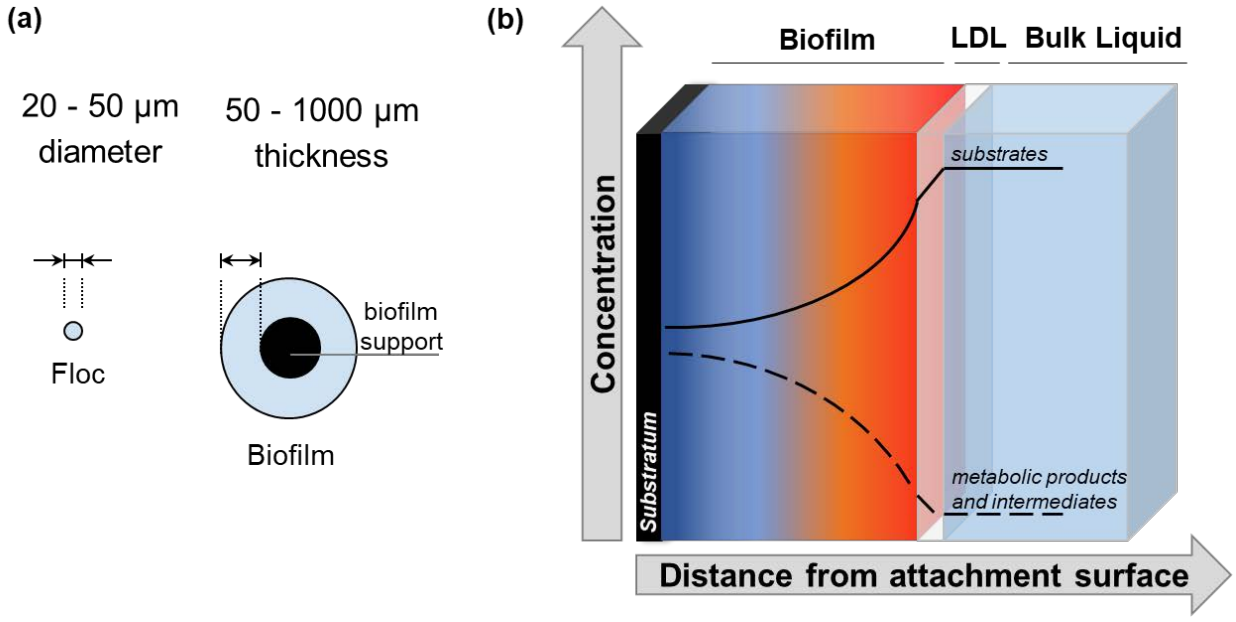
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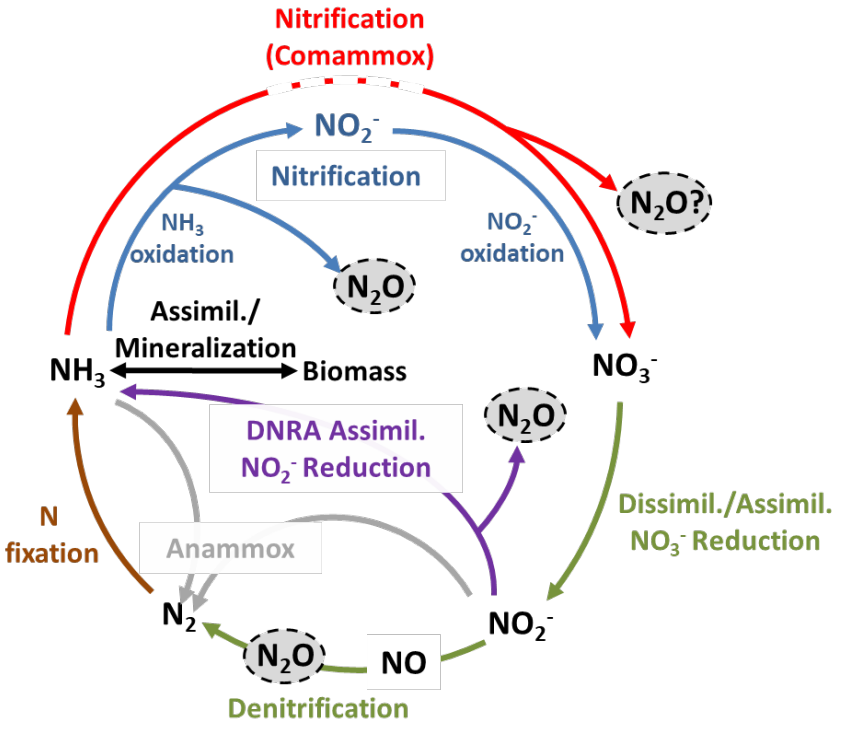
Nitrification
(Comammox)
NO_2^-
Nitrification
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DNRA Assimil.
NO_2^- Reduction
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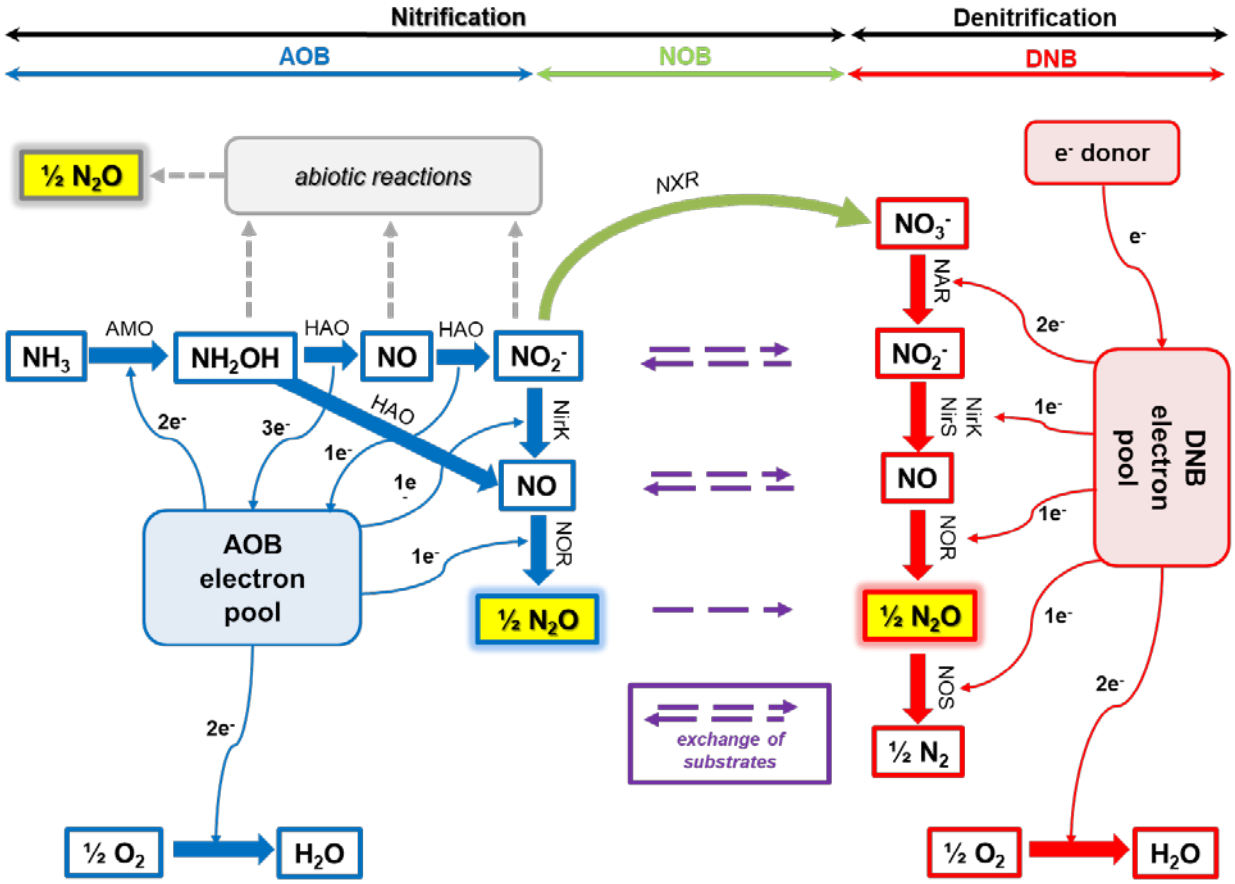

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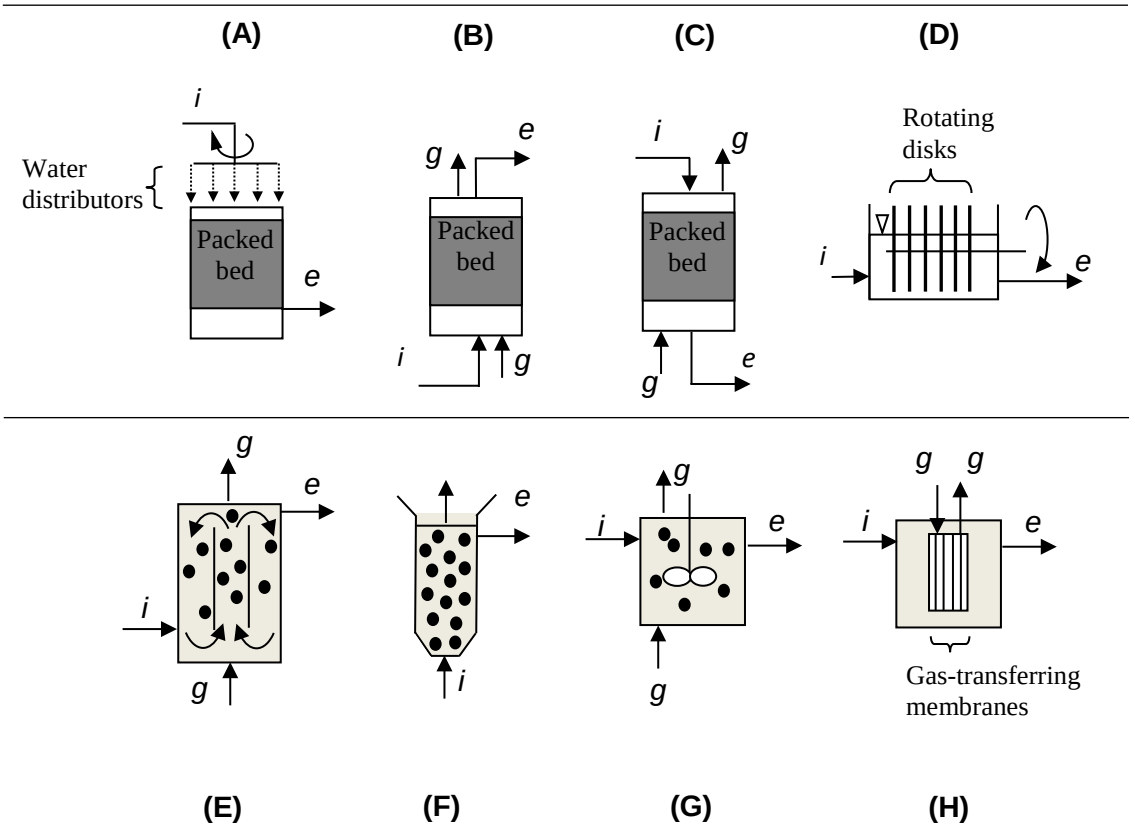
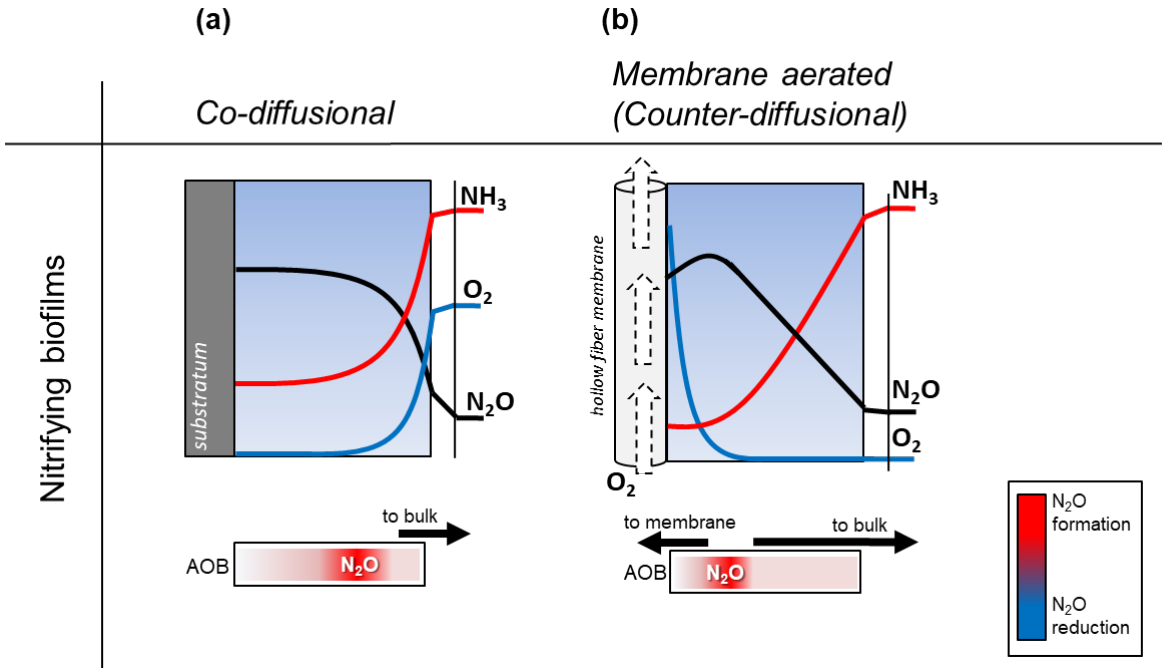
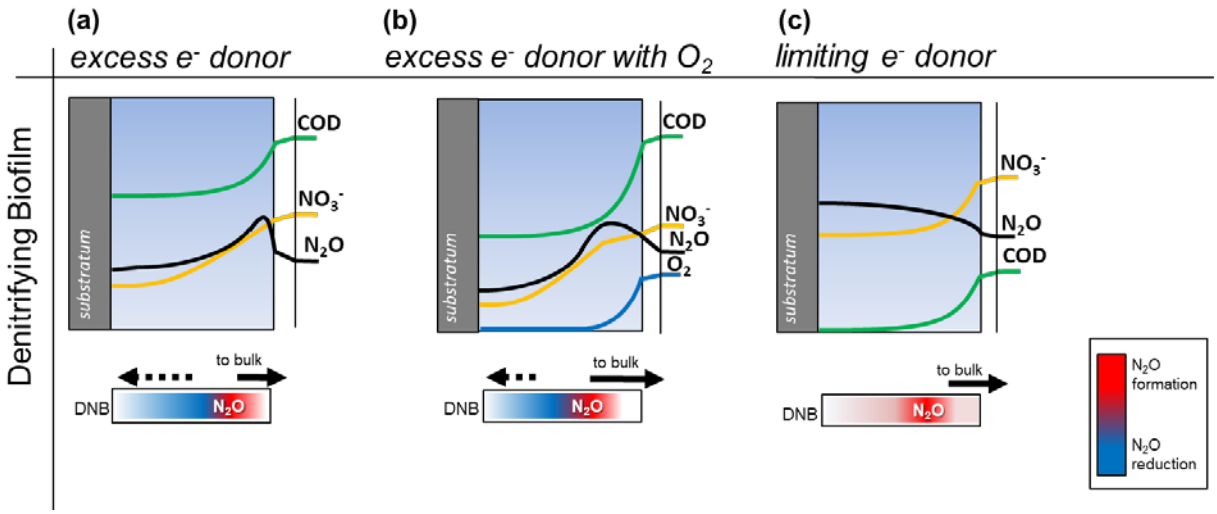

(A)
(B)
(C)
(D)
Water distributors
Packed bed
Rotating disks
(E)
(F)
(G)
(H)
Gas-transferring membranes


(a)
(b)
Co-diffusional
Membrane aerated
(Counter-diffusional)
Nitrifying biofilms
substratum
NH3
O2
N2O
to bulk
AOB
N2O
hollow fiber membrane
NH3
N2O
O2
O2
to membrane
to bulk
AOB
N2O
N2O formation
N2O reduction

1124

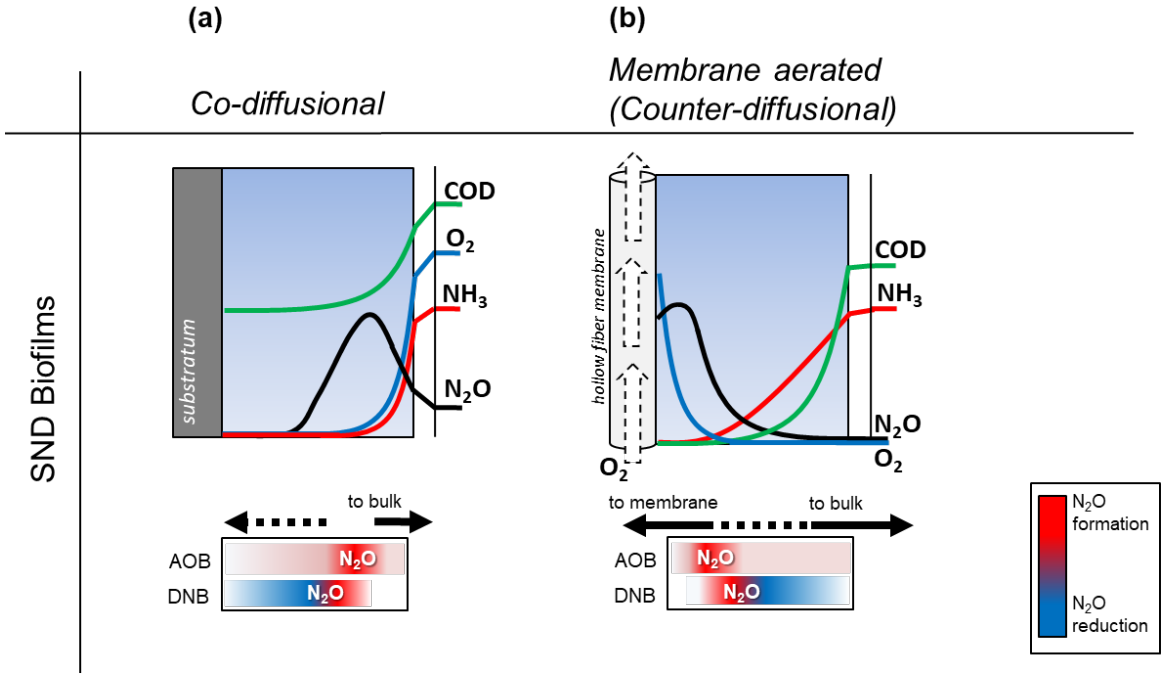
1125



1126

1127

1128



1129

1130

1131